The *Mycobacterium avium* subsp. *Paratuberculosis* Fur Element's Interactions with an *in vivo* Environment

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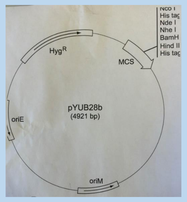
ABSTRACT

Johne's disease is a chronic digestive disease in dairy cattle that is caused by the bacteria, *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Currently, there is no effective vaccine for Johne's disease, and the detection testing for the disease is a long process. In order to shorten the testing and create a vaccine, we need to better understand how MAP interacts with its environment. Like most living organisms, MAP needs iron to survive, and it uses mycobactin for iron uptake. The ferric uptake regulator, Fur element, a gene on a horizontal gene transfer island unique to MAP, is likely utilized *in-vitro* to regulate an alternate iron acquisition pathway. To further study MAP's Fur element, we undertook studies to first demonstrate that the Fur box was functional in MAP. MAP3773C, or the Fur of MAP, was cloned into *M. smegmatis* on a MSMEG optimized vector. The colonies were grown, and protein production was induced *in vitro*. A sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was applied to determine protein expression and a Western Blot was used for confirmation. If MAP3773C was produced by the clones, an electrophoretic mobility shift assay (EMSA) was used to study the interaction of Fur to its binding sites or motifs. The starting method of protein expression was not successful, so different methods were studied with still no fur protein expression. Further work needs to be done with the protein expression. Optimization of the EMSA test was undertaken to get DNA-Fur protein binding to occur. The modified EMSA test shows DNA-protein binding occurs. In future studies, to further study the EMSA results, DNase footprinting and chromatin immunoprecipitation (ChiP-seq) will be used to identify the regulon of Fur.



MSMEG optimized vector used for cloning MAP3773C. This vector was used and MAP3773C was inserted into the vector. The His tag portion of the genome was used in the Western Blot test to see if the Fur protein was being expressed. Photo credit: Fernanda Shoyama

BACKGROUND

Mycobacterium avium subps. Paratuberculosis (MAP) is the bacteria responsible for Johne's disease, a chronic enteric disease of dairy cattle. The disease is contagious and untreatable, and is associated with large economic losses to the agriculture industry. There are tests for Johne's, but the testing is a long, slow process that interferes with timely implementation of mitigation strategies.

Iron as a micronutrient

- Almost all living organisms, bacteria included, need iron for reactions that are required for living.
- Mycobacteria use mycobactin for iron uptake and metabolism.
- MAP does not produce mycobactin *in vitro*, so the media needs to be supplemented with mycobactin, a likely cause of a slow growth rate.
- The Iron dependent repressor (IdeR) regulates the metabolism of MAP.

The Fur Element

- The Fur element is a transcriptional regulator that is present on a MAP specific genomic island that was likely acquired via horizontal gene transfer.
- The fur element has been present in the MAP genome for years, indicating that it may be used and likely interacts with the central iron regulator, IdeR.

In order to study the fur element efficiently, MAP3773C was cloned into *M. smegmatis*. The cloned *M. smegmatis* colonies were harvested and studied in this experiment for protein expression.

RESEARCH QUESTIONS

Is the fur element utilized *in-vitro* to regulate an alternate iron acquisition pathway? Our focus is the promoter sites of Fur on MAP.

Fur protein was expressed in *M. smegmatis* and its activity was studied using EMSA.

MATERIALS AND METHODS

Protein Expression

- Previously in the lab, MAP3773C was cloned into a plasmid and the plasmid was electroporated into *M. smegmatis*.
- The colonies were grown on plates for four days, then streaked and added to 20 mL of LB low-salt media.
- Once the optical density reached 1.0, 1mL of the culture was added to 29mL of LB low-salt media. Two of these cultures were made for each colony.
- Once the optical density reached 0.4-0.5, one culture for each colony was induced with IPTG to increase protein production, the other was not induced.
- The cells were collected from the supernatant and were washed and lysed to prepare for the SDS – PAGE.
- SDS - PAGE was performed to determine the fur protein had been expressed

DNA Protein Preparation

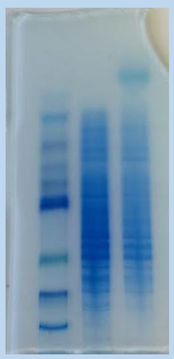
- **Extraction –** DNA was extracted from grown MAP K10 cells
- **Amplification -** Polymerase Chain Reaction (PCR) was used to amplify the promoter and the fur regions of the DNA. Used forward and reverse primers that selected for the promoters used in fur regulation. One primer was biotinylated to enable visualization of DNA-Fur binding in the EMSA.
- **Purification –** A Qiagen kit was used to purify the DNA obtained from the PCR.
- **Concentration -** The DNA was concentrated in a vacuum so the concentration of the DNA used would be higher.

EMSA

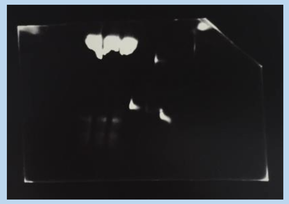
- Gel-electrophoresis was performed on the replicated DNA fragments with the fur protein to determine DNA-protein interaction. The Life Technologies product was used for the EMSA.
- There was a control set of reactions and 3 reactions to determine if the DNA-protein binding was occurring. The reactions were biotin DNA and no protein, biotin DNA and protein, and biotin DNA, non-biotin DNA, and protein.
- The gel was prepared for chemiluminiscent imaging using luminol as a substrate.
- The EMSA test is similar to the experiment done by Janagama et. al in 2010.

RESULTS

- More successful growth of the cloned colonies occurs when the colonies are grown on plates first, then inoculated into LB low-salt media.
- Fur was not expressed in the *M. smegmatis* using the growth and expression methods that were used. There was no protein band found at 15 kDa in the cell pellet or the supernatant.
- There is a shift in the EMSA when using a 6% gel and 0.5 µg of DNA with the fur protein.



Protein Expression – SDS PAGE example of results. There is no over expression at the 15 kDa band, meaning there was no Fur protein expression. A Western Blot was used to verify that there was no Fur protein expression. Photo credit: Kayla Brown



An EMSA test in the lab and an example EMSA test. The EMSA test on the left was unsuccessful in binding the protein to the DNA because there was no shift. The test on the right is what the gel should look like. There should be a shift in the gel in reaction 2 because the protein and DNA are bound together, so they move through the gel slower. In reaction 3 there should be less of a shift due to binding competition with the non-biotinylated DNA. Photo credit: left – Kayla Brown, right - Fernanda Shoyama.

DISCUSSION AND CONCLUSIONS

The protein expression was not successful because the MAP3773C protein, which is about 15 kDa, was not found in either the cell pellet or the supernatant. Some reasons for this could be that IdeR is being used to regulate iron uptake, so the Fur element is not needed, therefore the element was not produced. The iron concentration in the media may need to be altered so that there is more of a need for the Fur element. The lack of the Fur element on the genome could also be due to another gene that is interfering with Fur production.

The work done with the EMSA was performed to determine the protocol that will be used for future EMSA's done with *M. smegmatis* DNA that contains MAP3773C. The EMSA protocol will continue to change and become better suited for the DNA and protein of interest.

FUTURE WORK

Other methods of protein expression will be used for further study of MAP3773C. DNase footprinting assays will be used to further study the Fur binding sites shown in the EMSA by locating the specific binding sites of the proteins on the DNA.

We will use chromatin immunoprecipitation, or ChiP sequencing, to study the presence of the Fur binding sites on the genome and to identify the binding site's regulon. From this data we will be able to see which genes are more expressed when there is a higher iron concentration in the environment, leading to genes that communicate with the fur element.

ACKNOWLEDGEMENTS

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